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Artificial nerve guides

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CHAPTER 3

Long-term evaluation of functional nerve recovery after reconstruction with a thin-walled biodegradable poly(DL-lactide-ε-caprolactone) nerve guide, using walking track analysis and electrostimulation tests

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Abstract

This study was performed to evaluate the long-term functional nerve recovery after reconstruction of a 10 mm gap in the sciatic nerve of the rat with a thin-walled nerve guide, composed of a biodegradable copolymer of DL-lactide and ε–caprolactone [p(DLLA-ε-CL)]. To evaluate both motor and sensory nerve recovery, walking track analysis and electrostimulation tests were carried out after implantation periods ranging from 3 to 52 weeks postoperatively. The first signs of both motor and sensory nerve recovery could be observed after 5 weeks. After 15 weeks, 70% of the sciatic function and 90% of the sensory nerve function had been recovered. After this period, the sciatic function index (SFI) did not improve further, whereas the sensory nerve function appeared to return to normal. When the results of the SFI measurements, minus those obtained from rats with severe automutilation, are extrapolated, further improvement of the SFI might be expected after 52 weeks. The fact that 100% sensory nerve recovery was obtained, as measured by the electrostimulation test, could be explained by sensory reinnervation from surrounding areas. The SFI was not fully reestablished because automutilation had a great impact on the use of walking track assessment.

Introduction

The most widely used technique for the reconstruction of a peripheral nerve gap is the use of autologous nerve grafts. The donor nerve is usually obtained from nerves that are functionally less important, such as the sural nerve. This technique, however, has some disadvantages: there is loss of donor nerve function and the risk of neuroma formation at the donor site. One successful alternative to eliminate these problems is the use of biodegradable nerve guides.[1-3] After functioning as a temporary scaffold for nerve regeneration, they gradually degrade. The use of a biodegradable nerve guide composed of an amorphous copolymer of DL-lactide and ε-caprolactone [p(DLLA-ε-CL)] has proven to be effective.[4-6] Nerve regeneration across a 10 mm nerve gap, using a biodegradable nerve guide, was faster and qualitatively better, when compared with nerve regeneration through an autologous nerve graft.[7] Moreover, this nerve guide degrades fast and completely within 1 year.[6]

A nerve guide should have an internal diameter large enough to overcome problems when telescoping the nerve stumps into the lumen of the nerve guide during the implantation procedure. It should also have a thin wall that swells little during degradation and causes no nerve compression. However, with a too large internal diameter, it is more likely that fibrous tissue grows into the lumen of the nerve guide, thereby hampering nerve regeneration and maturation. Den Dunnen et al.[5,7] used biodegradable nerve guides with an internal diameter of 1.5 mm and a wall thickness of 0.3 mm, which have functioned optimally in a rat

model. To overcome the pronounced swelling during the degradation of p(DLLA-ε-CL), a nerve guide with an internal diameter of 1.4 mm and a wall thickness of 0.17 mm was implanted.

Full restoration of sensory and motor nerve function after peripheral nerve reconstruction often fails.[8] Evaluation of functional nerve recovery in the rat model is not simple. One way to evaluate the recovery of sensory function of the sciatic nerve is the withdrawal test, originally described by Young and Medawar.[9] One way to provoke a withdrawal response is by stimulating the footsole with an electric current (the so called electrostimulation test), as was described by De Koning et al.[10] and used by others.[11-14] Rat walking track analysis for the assessment of sciatic nerve function has been well documented following nerve injury and repair. Since the introduction of the walking track analysis in the rat by de Medinaceli et al.,[15] this form of analysis is increasingly being used.[16-18] Furthermore, due to the modification of the formula by Bain et al.,[19] walking track analysis is extremely reliable. However, **long-term** evaluation of functional nerve recovery using walking track analysis and electrostimulation tests is scarcely performed.[20]

The aim of this study was to evaluate long-term functional nerve recovery after reconstruction of a 10 mm gap in the sciatic nerve of the rat, using a thin-walled biodegradable p(DLLA-ε-CL) nerve guide. To evaluate both motor and sensory nerve recovery, walking track analysis and electrostimulation tests were carried out after implantation periods ranging from 3 to 52 weeks postoperatively.

Materials and Methods

Preparation of the Nerve Guides

The biodegradable nerve guide in this study was composed of a copolymer of 50% DL-lactide and 50% ε-caprolactone. The lactide component contained 85% L-lactide (LLA) and 15% D-lactide (DLA). The average molecular weight (Mw) was 1 x 10^6 kg/kmol and the polydispersity index was 2.5.

A solution of 3 wt% of the amorphous copolymer in chloroform was prepared. This solution was dip coated on a glass mandrel with a diameter of approximately 1.6 mm, as described in detail by den Dunnen et al.[5] This technique resulted in a nerve guide with an internal diameter of 1.4 mm and a wall thickness of approximately 0.17 mm.

After preparation, the nerve guides were stored in 100% ethanol at 4 °C. Before implantation, the nerve guides were first washed in 0.1 M sterile phophate-buffered saline (PBS) at room temperature, and then filled with 0.1 M sterile PBS.

Surgical Procedures

Male Wistar rats (n = 20), weighing approximately 250 g, were premedicated with atropine (0.25 mg/kg body weight) and anesthetized with 1% isoflurane (Forene®, Abbott Laboratories Ltd., Queenborough, UK) and O_2/N_2O. The left sciatic nerve was exposed through a superficial gluteal muscle-splitting incision. A 7 mm segment was then resected, leaving a gap of about 10 mm due to retraction of the nerve ends. Continuity was reestablished using a 12 mm nerve guide. During the implantation of the nerve guide, both the proximal and distal cut ends of the sciatic nerve were tele-

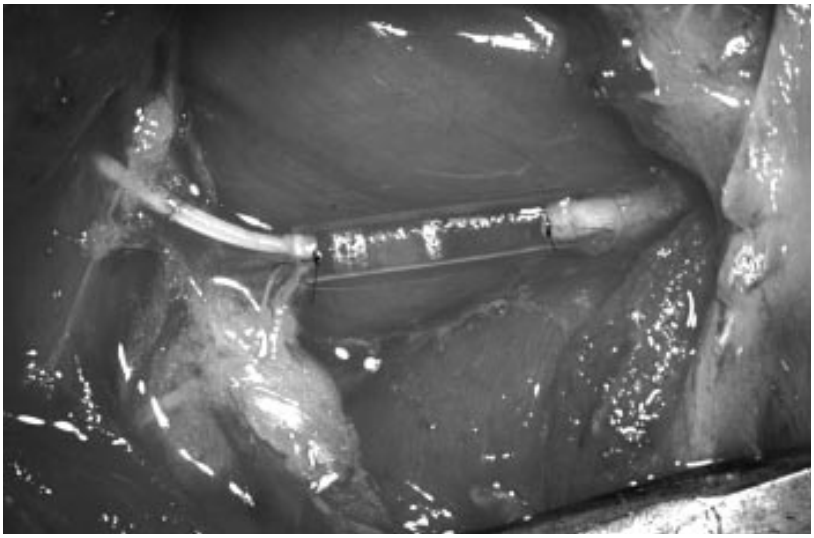

Fig. 1. *Photograph of an implanted thin-walled biodegradable poly(DL-lactide-ε-caprolactone) nerve guide, as was used in this study. Both the proximal and distal nerve stumps were telescoped into the lumen of the nerve guide and fixed with a single stitch. The length of the nerve guide is 12 mm.*

scoped into the ends of the nerve guide and fixed with a single 9-0 nylon epineural suture [Auto Suture (ussc), MV 100-4 needle] (Fig. 1). Surgical procedures were performed under an operation microscope (Zeiss OPMI-6, Weesp, The Netherlands) and a sterile technique was used throughout the procedure.

After surgery, the animals were housed in a temperature- and humidity-controlled room with 12 hr light cycles and had access to standard rat food and water ad libitum. Good laboratory practice (GLP) was observed, according to the National Guidelines for Animal Welfare, comparable with the international rules for animal experimentation (International Guide on Animal Biomedical Research and Ethical Code for Animal Experimentation of the Council for the International Organization of Medical Sciences).

Walking Track Analysis

After 3, 5, 8, 15, 21, 26, 34, 43 and 52 weeks of implantation, walking track analysis was carried out, as was described in detail by Meek et al.[14] From the footprints, several measurements were taken: distance from the heel to the third toe, the print length (PL); the distance from the first to the fifth toe, the toe spread (TS); and the distance from the second to the fourth toe, the intermediary toe spread (IT). All three measurements were taken from the left operated foot (OPL, OTS, OIT) and the contralateral nonoperated foot (NPL, NTS, NIT). As a result, corresponding factors (print length factor [PLF], toe spread factor [TSF], and intermediary toe spread factor [ITF]) could be calculated.[19] Incorporating these factors into the following equation derived by Bain et al.[19] the sciatic function index (SFI) can be calculated as follows:

SFI = -38.3 x PLF + 109.5 x TSF + 13.3 x ITF -8.8 (I)

An SFI of 0 is normal. An SFI of -100 indicates total impairment.

The most representative prints were measured for each rat. Sometimes several walks were required to obtain clear print marks. Furthermore, the walking track measurements were repeated and the resulting SFIs averaged. As a control, we took the SFI value for a perfect functioning hindpaw; SFI = 0.

Electrostimulation Tests

After 3, 5, 8, 15, 26, and 52 weeks of implantation, electrostimulation tests to evaluate sensory nerve recovery was carried out, as was described in detail by Meek et al.[14] The nonoperated contralateral footsole served as a control. An electrical stimulator with an adjustable current between 0 and 1.0 mA was used for this purpose. A healthy rat will immediately withdraw its foot and spread its toes upon skin contact with the electrodes. The threshold, i.e. the lowest current causing this reflex, was recorded and the mean values evaluated.

Macroscopy

During this 52-week study, automutilation (only at the operated site) of the rats' hind feet was observed. Therefore, the influence of automutilation on the outcomes on the long-term results of the walking track analysis was evaluated. The paws of the rats were examined for signs of automutilation. Superficial wounds restricted to the nails or the cutaneous part of the rats' hindpaws were indicated as moderate, whereas more extensive wounds (as exposed bone or the absence of a part of the paw) were scored as severe (Fig. 2A+B).

Results

Walking Track Analysis

The SFIs (Equation I), as applied to the measurements of the walking track patterns, are shown in Figure 3. Preoperative SFI values for the test group did not differ from the control values. The first signs of sciatic nerve recovery could be observed after 5 weeks (SFI -81; SD ± 10.6). After 15 weeks, an SFI of -33 (SD ± 5.4) was found. After this period, no further improvement of the SFI was found and after 52 weeks, the SFI was –46 (SD ± 28) (line 2). During this 52-week study, two phases could be observed. During phase 1, there was improvement in the SFI; during phase 2, there was no further improvement of the SFI.

Severe automutilation negatively influences the average SFI in the long term (line 1). The SFI increases to approximately -30 after 52 weeks of reconstruction. In the second phase, the SFI still increases and does not reach a plateau. It can also be observed that in the case of automutilation, SFI values decrease in the long term (line 3).

Electrostimulation Test and Macroscopic Evaluation

The current (mA) necessary to cause a withdrawal reflex of the foot is outlined in Figure 4. In the first 3 weeks after implantation of the nerve guide, the maximum current of 1.0 mA was not enough to cause a withdrawal reflex. After 5 weeks, the first signs of sensory nerve recovery could already be observed. Af-

ter 8-15 weeks, the threshold decreased sharply to 0.27 mA. After 26 weeks, the threshold returned to normal (control value). This value was also observed 52 weeks after reconstruction. The threshold of the contralateral control foot was 0.19 mA (strait line in Fig. 4).
Automutilation was not observed at the evaluation points of the electrostimulation test, but was restricted to superficial cutaneous wounds more medial from the evaluation points and the absence of the nails and parts of the third, fourth and fifth toes. During this study, the total number of rats decreased with time, because rats were sacrificed (selected at random) for histological analysis. The percentage of rats with signs of

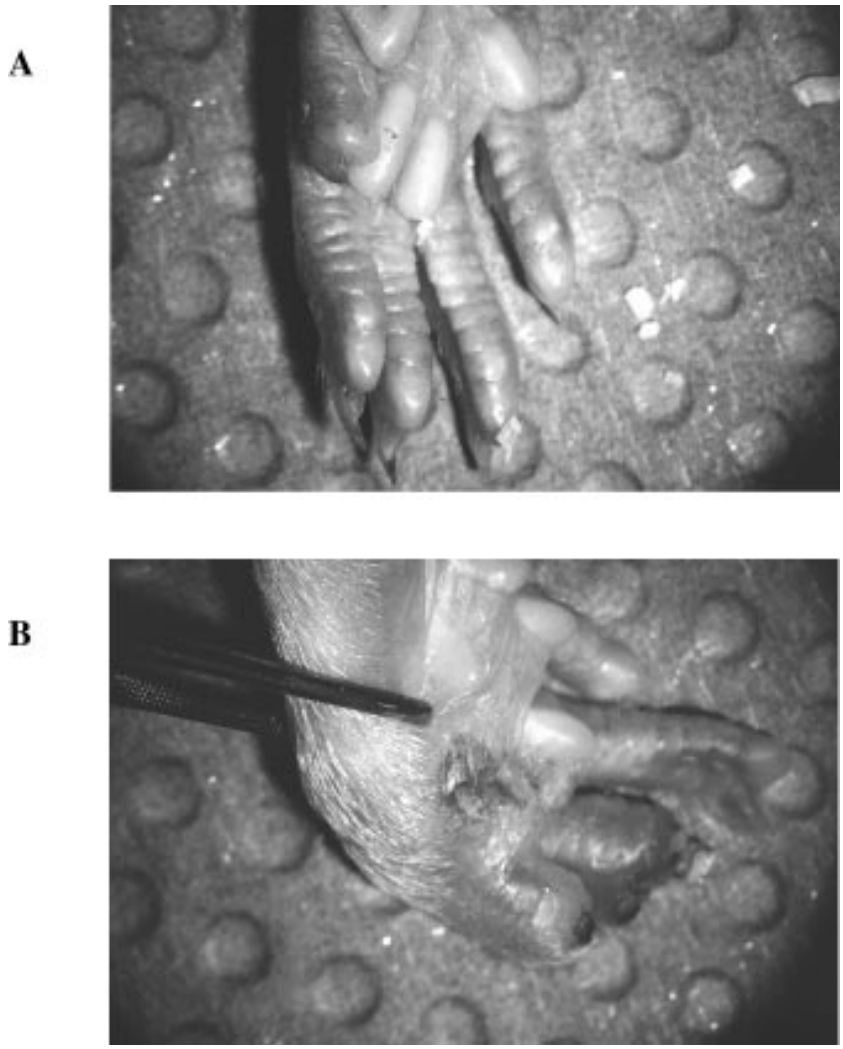

Fig. 2. *Photographs showing the dorsal sides of the rats' hindpaws 52 weeks after implantation of the thin-walled biodegradable nerve guides.* ***A****: Intact hindpaw.* ***B****: Severe automutilation: absence of parts of the fourth and fifth toe, absence of nails, and a cutaneous wound more cranial from the fifth toe.*

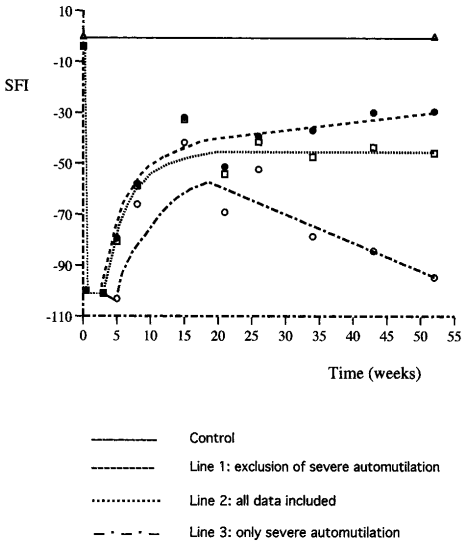

Fig. 3. *Graph showing changes in the average Sciatic Function Index (SFI) as a function of time. Note that an SFI of 0 is normal, whereas an SFI of -100 indicates total impairment. After 3 weeks the SFI starts to increase to -33 after 15 weeks. After this period, the SFI did not further increase (line 2). When the rats with automutilation are excluded (line 1), an increase in SFI can be observed. It can also be observed that in the case of automutilation, SFI values decrease on the long term (line 3).*

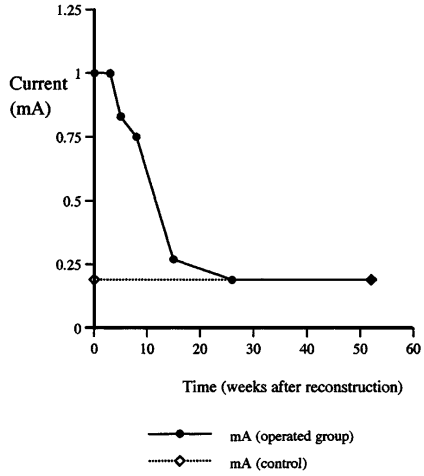

Fig. 4. *Graph showing the change in the average current necessary to cause a withdrawal reflex of the foot of the rat with time. The current returned to normal after 26 weeks.*

automutilation relatively increased with time, which in turn led to a relative decrease in the percentage of rats without any form of automutilation (Table 1). In two automutilated rats, the formation of contractures was observed.

Discussion

Immediately after transection of a sciatic nerve, an impaired gait and walking track can be observed: loss of ankle plantar flexion, foot inverters, toe flexors and foot intrinsics are characteristic. The footprints then demonstrate an increased PL, decreased TS, and decreased IT.[19] Complete loss of function can then be observed, as depicted by corresponding SFI values near -100.

Some problems with the inter- and intraobserver reliability and the validity of the walking track analysis measurement technique have been described.[17] To overcome these problems and to obtain more significant data in this study, several prints from each rat were measured by one observer. Until 15 weeks after reconstruction, sciatic nerve function increased from an SFI of -100 to -33 (e.g., a 70% improvement). However, after this period no further improvement in SFI was seen (phase 2).

Information about long-term functional nerve recovery after sciatic nerve reconstruction using walking track analysis is scarce. Hare et al. [18] described functional nerve recovery over a 1 year period following fascicular sciatic nerve repair using epineural sutures. They found an SFI of -102.4 after 2 weeks and an SFI of -59.0 (e.g., a 41% improvement) after 8 weeks (phase 1). The SFI values never recovered to control values: after 52

Table 1. *Total number of rats and the number and percentage of rats with no, moderate, and severe hindpaw automutilation during the evaluation period. Note the relative increase of automutilation on the long term, due to decrease of the number of rats on the long term.*

weeks after reconstruction	n rats	none n (%)	moderate n (%)	severe n (%)
0	20	20 (100)	0 (0)	0 (0)
3	20	20 (100)	0 (0)	0 (0)
5	17	14 (82)	2 (12)	1 (6)
8	14	10 (71)	2 (14)	2 (14)
15	11	8 (73)	2 (18)	1 (9)
21	8	5 (63)	2 (25)	1 (13)
26	8	5 (63)	2 (25)	1 (13)
34	4	3 (75)	0 (0)	1 (25)
43	4	3 (75)	0 (0)	1 (25)
52	4	3 (75)	0 (0)	1 (25)

weeks, Hare et al. described an SFI of -80.1 ± 22 (phase 2). In this study, an SFI of -46 was found (Fig. 5). In the study of Hare et al. and in the present study, two phases during the 52 weeks of evaluation were experienced (Fig. 5): in phase 1, there was an improvement in the SFI and in phase 2, there was no further improvement in the SFI. The second phase in the present study starts later than in the study of Hare et al. This can be explained by the fact that in our study a 10 mm gap needs to be bridged, whereas in the study of Hare et al. no gap had to be bridged. It can be observed that in our study, relatively good results were obtained compared with the results of Hare et al.[18] Furthermore, it can be observed that in the present study phase 2 (the plateau phase) reaches a higher SFI value than in the study of Hare et al., probably be due to a faster and better nerve regeneration with less intraneural fibrosis.

Abnormal walking track patterns influence the mean SFI values. In the study of Hare et al., [18] return of function by SFI measurements was negatively influenced by contracture formation. Dellon and Mackinnon found that at a time longer than 1 year following nerve repair, chronic foot deformities result in gait patterns that render walking track assessment invalid.[20] In addition, they concluded that according to morphometric analysis, reinnervation of the leg and foot musculature should occur. However, there is always a significant misdirection of the degenerating nerve fibers, resulting in muscle imbalance. In our study, the SFI was also negatively influenced by severe automutilation of the rats' hindpaw, especially in the long term (as was shown in Fig. 3), since the mean SFI was based on relatively less rats. The SFI of rats without automutilation, however was good, and in the case of automutila-

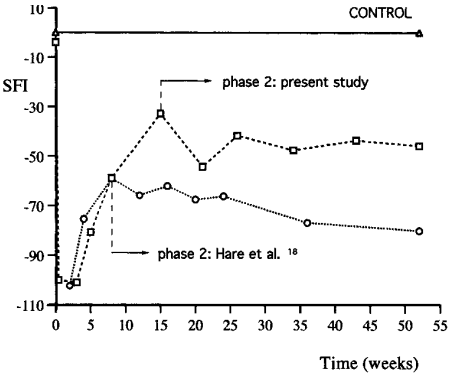

Fig. 5. *Graph showing changes in the average Sciatic Function Index (SFI) with time of the present study and the study of Hare et al.[18] Note that in both studies two phases can be distinguised; improvement and no further improvement of the SFI.*

tion, SFI values decreased in the long term.

Scott also reported possible explanations for the fact that SFI values do not recover to control values.[21] According to Scott, poor quality of nerve recovery following nerve transection may be due to: (1) the failure of regenerating axons to cross the junction between the proximal and distal nerve stumps and (2) the mislocation of the axons within the peripheral target regions (cross-innervation). The axons that do successfully regenerate across the nerve gap are unlikely to reinnervate their original target site or even the original region. Therefore, the muscle and tendon receptors may be reinnervated by inappropriate afferent axons. The motor unit organization may also be altered by clumping of the muscle fibers.[22] This leads to changes in the force distribution profiles during muscle contraction, which in turn will significantly affect the mechanical input to the tendon organs.[23] The mislocation of the afferent axons within the peripheral target regions and the uninnervated receptors have a disadvantageous effect on the utilization of proprioceptive feedback in movement control, as reflected in the walking track patterns. This phenomenon may be responsible for the fact that 15 weeks after implantation, no further improvement in the SFI was found. However, on the basis of line 1 in figure 3 (SFI minus SFI of rats with automutilation), an increase in SFI can be observed. When the results of the SFI measurements, minus those obtained from rats with severe automutilation, are extrapolated, it could be concluded that further improvement in the SFI might be expected after 52 weeks.

In the clinical situation, patients have to cope with an altered input of information and they have to "reprogram" the brain through reeducation programs. There are indications that this reprogramming of the brain involves phenomena such as (1) long-term potentiation of existing synapses, (2) the formation of new synapses, and (3) probably the expression of new proteins in the cortical cells. All these processes together are called "cortical plasticity".[24] In the case of rats in this study, reprogramming of the brain can explain the improvement in the SFI in the long term. Whether the SFI reaches control values after longer periods, however, remains uncertain.

The local application of small electrical stimuli to the rat's footsole is a noninvasive, rapid, easy, and very precise method to evaluate the return of sensory nerve function after nerve injury.[10] Other techniques have been described in the past, but they were not useful to evaluate the different areas of the footsole of the rat,[25] or they were too subjective.[26]

The electrostimulation tests can also be used for longitudinal research to evaluate the speed of sensory nerve function, while there is no conditioning during the test.[10] When a sensory nerve is sectioned, the corresponding area of skin becomes anesthetic. Thereafter, sensory nerve recovery occurs in an orderly sequence.[27] Van Meeteren et al.[11] evaluated functional nerve recovery after sciatic nerve crush lesions in the rat and they also found a difference in return of motor and sensory nerve function. They performed motor and sensory nerve conduction velocities (MNCV and SNCV, respectively) by which the crushed nerve was directly stimulated. The MNCV started to recover earlier than the SNCV. Moreover, 21 weeks after injury, the MNCV returned to 70% of the control value, while the SNCV returned to approximately 50%. The results of the conduction velocity, however, do not provide information on whether the nerve fibers have reached their distal targets or not. The conduction velocity is defined by the axon diameter.[28] In general, the sensory axons are thicker and, therefore, regeneration takes more time than in motor axons. In this study, however, we used electrostimulation tests to evaluate the sensory nerve recovery, which also stimulates the smaller axons. The electrostimulation test is used as a rough assessment of sensory nerve recovery.

The fact that the sensory nerve recovery was fully reestablished, as measured by the electrostimulation test, in the present study does not guarantee the single-handed outgrowth of fibers from the sciatic nerve. The mechanism behind the return of sensory nerve function can be explained by outgrowth of regenerating axons from the proximal sectioned sciatic nerve stump, but also by collateral sprouts emitted by intact fibers in the skin surrounding the denervated zone,[29] or a combination of both. No conclusions about these mechanisms can be drawn from the present study. However, trophic factors such as nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF), which accumulate inside nerve guides are known to induce collateral sprouting from (non)injured axons.[30] Collateral sprouting may also occur from intact axons and subsequently make functional peripheral connections.[31] Furthermore, Devor et al.[26] showed that the early phase of return of sensation in the foot, following sciatic nerve crush, is accounted for by collateral expansion of the functional distribution of intact neighboring fibers of the saphenous nerve. Moreover, they showed that this reinnervation is later replaced upon regeneration of the original nerve, while the saphenous nerve retreats from its maximal extent and returns very nearly to its original boundaries. Whether this is also the case in this study has not been investigated. To evaluate this phenomenon, a simple test can be performed: after stimulating, the saphenous nerve is transected and the electrostimulation test repeated. The region of expansion of fibers originating from the saphenous nerve can be outlined in this manner.

In conclusion, sensory nerve function, as measured by the electrostimulation test, fully recovered after reconstruction of a 10 mm nerve gap with a biodegradable p(DLLA-ε-CL) nerve guide. Recovery of the sciatic nerve function occurred in two phases. Until 15 weeks after reconstruction, an increase in SFI was found,

whereas after this period the SFI did not further improve. Further improvement in the SFI after 52 weeks can be explained on the basis of cortical plasticity.

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